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13. ABSTRACT (Maximum 200 Words)

The purpose of this project was to search for mutations that affect susceptibility to breast cancer by screening the progeny of specially-bred mice. The male parent in each case had been treated with ethylnitrosourea (ENU) in the hope of inducing mutations. The female parent was homozygous for the c-neu oncogene under the control of the mouse mammary tumor virus (MMTV) promoter. In previous experiments, female mice that carry the MMTV-neu gene expressed a very high frequency of breast cancer after pregnancy and lactation, which are known to activate the MMTV-neu oncogene. After screening the progeny of 68 treated males, no mutations that affect breast cancer were identified, although mutations that affected other organ systems occurred. However, the major finding of the study was related to one of the control strains. The MN-10 strain carries the MMTV-neu oncogene on the BALB background and had previously demonstrated fairly high susceptibility to breast cancer at our institution. In the current project, its breast cancer rate was very low, and we believe this can be attributed to changes in animal care conditions that occurred since the previous work. The significance of this study was the identification of an ideal mouse model for studying environmental effects on spontaneous mammary tumors.

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Introduction

The strategy for this project was based upon the concept that cancer is the end result of a series of genetic changes, and that most of these changes involve somatic mutations in the developing tumor. It had been well established that certain strains of mice that carry genes that are believed to be involved in human breast cancer do indeed have a higher than normal rate of breast cancer. One such strain is known as MN-10, which has the BALB background but carries the neu oncogene under the control of the mouse mammary tumor virus (MMTV) promoter. We proposed that if an additional mutant gene that is relevant to breast cancer were introduced into the same line, the rate of breast cancer would go even higher. We therefore proposed to treat male mice with N-ethyl-N-nitrosourea (ENU) to induce mutations, and then mate the treated males with MN-10 females. We would then examine the female progeny of these mice, after pregnancy and lactation to turn on the MMTV promoter, for their susceptibility to breast cancer. If these females had inherited a relevant mutation from the treated male, they should be more susceptible than control MN-10 females.

Body

The MN-10 strain that we used was descended from the same mice that had previously been used to study the role of the $p185^{neu}$ receptor in the development of breast cancer (Katsumata et al., 1995). In that study, approximately 90% of the control females developed mammary tumors by one year of age. Females of this strain were mated with BALB males that had been treated with ENU according to previously published methods (Justice et al., 2000). It is well known that a temporary period of sterility occurs after ENU treatment, but the BALB males in our experiment appeared to be more sensitive to the effects of ENU than what had been reported. We therefore (a) modified the dose of ENU, and (b) supplemented the numbers by treating males of the FVB strain with ENU and mating them with partners of the FVB-TgN(MMTVneu) strain, line #202 (Li et al., 1997). This strain also carries a mutant c-neu oncogene under the control of the MMTV promoter, but the background strain, FVB, is well known to be more robust and fertile than BALB. Task 1, to obtain hybrids between ENU-treated males and female partners that carry the MMTV-neu gene, was completed by the end of the first year, with slight modifications from the original plans.

Task 2 was to observe the female carriers for 6 months. Because no tumors appeared during the first 6 months, this time frame was extended. Female mice with the relevant genotypes that had borne litters (to activate the MMTV promoter) were maintained until the funds were depleted. For some females this was well over a year, but for those that were born later in the project, the time was necessarily shorter. During this period of observation, one of the lines developed abnormalities of the spleen and lower G.I. tract that appeared to be the result of a mutation. The abnormality appeared to be inherited as a dominant, but it was not pursued further as it did not fall under the purview of the original aims.

The first mammary tumor that we observed was in a control MN-10 female that was 17 months of age. We subsequently observed a second mammary tumor in a control MN-10 female at 12 months of age. The third tumor we observed was in the daughter of a treated male. This mouse was 11 months old when the tumor appeared. However, none of the other 37 females in this line that were positive for MMTV-neu developed a mammary tumor, so the one tumor that was observed could not be attributed to an ENU-induced mutation.

As the project progressed, it became obvious that the control mice, untreated MN-10

females, were not developing tumors at the same frequency as previously reported from our own institution. Data from the current project, in which mice were kept in specific pathogen free (SPF) facilities compared to previous data (collected when conventional facilities had been used) are shown in Table 1. Changes in animal care facilities are discussed below.

Table 1. Numbers of mice that had breast tumors and did not have tumors by 52 weeks of age in conventional facilities vs. SPF facilities.

	1993-1995 Conventional Facilities	2000-2003 SPF Facilities
Tumors	**20**	**1**
No Tumors	**3**	**10**
	X^2 = **19.1**	**P < 0.0001**

If we set an earlier endpoint, we can include some MN-10 mice that are still alive, but are not yet one year of age.

Table 2. Numbers of mice that had breast tumors and did not have tumors by 45 weeks of age in conventional facilities vs. SPF facilities.

	1993-1995 Conventional Facilities	2000-2003 SPF Facilities
Tumors	**19**	**0**
No Tumors	**4**	**20**
	X^2 = **29.6**	**P < 0.0001**

There were only two possible explanations: something had changed genetically in this strain, or there had been a relevant environmental change. With regard to the first possibility, any mutation that would have altered the expression of the c-neu oncogene could have had an effect of this type. We therefore tested for expression of the c-neu oncogene by RT-PCR. The results clearly showed that the c-neu gene was expressed in MN-10 mammary tissues at a very high level, as in previous experiments.

The other possible explanation for these results is that an environmental change occurred that was relevant to the development of mammary tumors. This would include possible differences in infectious agents, diet, or other factors. After the previous project (Katsumata et al., 1995) had been completed and before our current project commenced, our institution carried out extensive renovations in its animal care facilities, and adopted specific pathogen-free (SPF) policies. It was necessary to re-derive the MN-10 line by caesarian delivery in order to continue maintaining this strain in our facilities. Veterinary records from the old facilities were consulted, and it was determined that the following had all been identified in the mouse colonies at that time: Sendai virus, MHV (Mouse Hepatitis Virus), MVM (Minute Virus of Mice), PVM (Pneumonia Virus of Mice), Mouse adenovirus, and GDVII (a variant of Theiler's Virus). Rats that were housed nearby had been positive for SDAV (Sialodachroadenitis Virus), Mycoplasma pulmonis, GDVII, pinworms, Sendai Virus, and Pseudomonas aeruginosa. We considered the possibility that one or more of these agents may been relevant to the susceptibility of the mice to breast cancer.

It is well established that infection with *Helicobacter pylori* is relevant to the development of gastric cancer (Hiyama et al., 2002; Kim et al., 2002). Although this may seem to be a unique case and not especially relevant to breast cancer, it has been proposed on the basis of epidemiological data that breast cancer should be considered an environmental disease (Sasco, 2001). Specific mechanisms that could account for a connection between inflammation and breast cancer have been proposed by Coussens and Werb (2001).

A somewhat different environmental explanation would involve dietary or hormonal changes, which could have been associated in some way with differences in the animal care procedures. A report was recently published which demonstrated an effect of dietary estrogens on breast cancer development in an MMTV-*neu* mouse model (Yang et al., 2003). These investigators divided 338 FVB/N-TgN(MMTV-*neu*) mice into two groups. One was fed a conventional laboratory diet that included soy protein (Purina 5001), and other received a casein-based rodent chow that included no estrogenic activity. Mice were also implanted subcutaneously with slow-release pellets of estrogen, tamoxifen, or placebo. For placebo- and estrogen-fed groups, the mice that were maintained on a soy-based diet developed tumors at a significantly older age than the casein-fed controls. It is possible that a similar dietary change could have affected our results. The mice in the current project were fed TestDiet 5010, which includes a significant quantity of soy protein. As nearly as we can determine, the mice in the conventional facility received a similar diet during the 1990s, but our funds were not sufficient to repeat our study with a casein-based mouse chow.

Because we did not observe any ENU-induced mutations that were relevant to breast cancer, we were unable to carry out Task 3 or Task 4, which would have dealt with mapping the ENU-induced mutations.

The significance of this project is related to the interaction between genetic and environmental influences on breast cancer. The MN-10 strain carries a gene that gives it a

certain degree of susceptibility to mammary tumors. Under certain conditions (its current animal care environment), the breast cancer rate is quite low. Under certain other conditions (its former animal care environment), its breast cancer rate is very high. If the factor or factors that explain this difference were identified, this could have an impact on human breast cancer studies. Perhaps this is related to the epidemiological evidence for environmental effects on breast cancer susceptibility (Sasco, 2001).

Personnel

Dr. David L. Gasser was the Principal Investigator, and **Dr. Makoto Katsumata** was a collaborator who received partial salary support. A great deal of technical assistance was provided by **Tsai-Lung Tsai**, who was also supported by the grant. **Dr. Min Peng** was an important collaborator, but did not receive support from the grant.

Key Research Accomplishments

We have demonstrated that the MN-10 strain, which carries a mutant rat *neu* gene under the control of the MMTV promoter on a BALB background, has undergone a significant change in its susceptibility to breast cancer during the period of time that it has been maintained at the University of Pennsylvania. This strain has been maintained at the University of Pennsylvania for more than 10 years, and in a study that was conducted from 1993 to 1995, these mice were shown to develop breast cancer spontaneously at a high frequency. By 75 weeks of age, 100% of 23 mice had developed mammary tumors, with approximately 80% of the tumors occurring by 45 weeks of age. When the same strain of mice (i.e. descendents from those studied earlier) was studied from 2001 to 2003, a highly significant reduction in the frequency of mammary tumors was observed. None of 20 MN-10 females developed breast tumors by 45 weeks of age, and only one of 11 had developed a mammary tumor by the age of one year. The strain continues to express the *c-neu* oncogene as it did during the earlier study. The most significant environment change that occurred during this period of time was a change from conventional to SPF animal housing, and it is our hypothesis that something related to this change had an impact on breast cancer susceptibility.

Reportable Outcomes

A poster based upon this work was presented at the 2002 Era of Hope meeting.

Conclusions

We have demonstrated that the MN-10 strain of mice that is maintained at the University of Pennsylvania has undergone a highly significant reduction in its susceptibility to breast cancer during the past 10 years. One possible explanation for this is that a change from conventional to SPF housing led to the elimination of some viral or bacterial agent that had an impact on tumor susceptibility. Another possibility is that dietary changes may have been relevant.

The descendents of 68 male mice that had been treated with ENU were studied for evidence of ENU-induced mutations that have an effect on breast cancer. However, no such mutations were observed.

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Appendices

The abstract and copies from the poster that was presented at the 2002 Era of Hope Meeting are attached.

A SEARCH FOR MUTATIONS THAT AFFECT SUSCEPTIBILITY TO BREAST CANCER IN MICE

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In order to identify new genes that are involved in the induction and progression of breast cancer, we have adopted a strategy based upon mutagenesis by ethylnitrosourea (ENU), and mating with partners that are known to carry a transgene that confers a partial degree of susceptibility. Individual lines derived from these matings that have a significantly higher or lower degree of susceptibility to breast cancer than the control transgenics will studied to determine whether they carry an ENU-induced mutation that interacts with the transgene to affect susceptibility. In the first experiment, a transgenic line was utilized which carries the c-neu oncogene controlled by the MMTV promoter on the BALB/c background. None of these mice get mammary tumors before 6 months of age, but 35% of them have tumors by the age of 9 months. Our hypothesis is that tumor progression may be enhanced or delayed by ENU-induced mutations. Treated males have therefore been mated with transgenic females, and their progeny are being observed for the occurrence of mammary tumors. In the second experiment, a transgenic line that carries the p53-172H transgene under the control of the whey acidic protein (WAP) promoter on the FVB background is utilized. None of these mice develop breast tumors spontaneously, but bitransgenic mice that carry the c-neu oncogene in addition to the p53-172H transgene develop breast tumors at a median age of 154 days. The singly transgenic MMTV-neu mice with this background develop breast tumors at a median age of 234 days. Our hypothesis is that a similar acceleration of tumor development should be observable when the p53-172H transgene is coupled with an appropriate ENU-induced mutation. FVB males have therefore been treated with ENU, and have been mated with p53-172H transgenic females. In addition to searching for mutations identified on the basis of a phenotypic effect (increase or decrease in tumor susceptibility), we are also searching for new missense mutations identified by RT-PCR in genes known to be of interest.

The U.S. Army Medical Research Materiel Command under DAMD17-00-1-0398 supported this work.

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Abstract

In order to identify new genes that are involved in the induction and progression of breast cancer, we have adopted a strategy based upon mutagenesis by ethylnitrosourea (ENU), and mating with partners that are known to carry a transgene that confers a partial degree of susceptibility. Individual lines derived from these matings that have a significantly higher or lower degree of susceptibility to breast cancer than the control transgenics will be studied to determine whether they carry an ENU-induced mutation that interacts with the transgene to affect susceptibility. In the first experiment, a transgenic line was utilized which carries the c-neu oncogene controlled by the MMTV promoter on the BALB/c background. None of these mice get mammary tumors before 6 months of age, but 35% of them have tumors by the age of 9 months. Our hypothesis is that tumor progression may be enhanced or delayed by ENU induced mutations. Treated males have therefore been mated with transgenic females, and their progeny are being observed for the occurrence of mammary tumors. In the second experiment, a transgenic line that carries the p53-172H transgene under the control of the whey acidic protein (WAP) promoter on the FVB background is utilized. None of these mice develop breast tumors spontaneously, but bitransgenic mice that carry the c-neu oncogene in addition to the p53-172H transgene develop breast tumors at a median age of 154 days. The singly transgenic MMTV-neu mice with this background develop breast tumors at a median age of 234 days. Our hypothesis is that a similar acceleration of tumor development should be observable when the p53-172H transgene is coupled with an appropriate ENU-induced mutation. FVB males have therefore been treated with ENU, and have been mated with p53-172H transgenic females. In addition to searching for mutations identified on the basis of a phenotypic effect (increase or decrease in tumor susceptibility), we are also searching for new missense mutations identified by RT-PCR in genes known to be of interest

Introduction

It is generally agreed that in most cases, tumor progression occurs as a result of the interaction of a number of mutant genes that have occurred in the tumor cells. In order to search for previously unidentified genes that may be involved in the progression of mammary tumors, we have treated male mice with various doses of ethylnitrosourea (ENU) and mated them with female partners that are already carrying one mutant gene that is known to confer a certain degree of susceptibility to breast cancer. The aim is to observe the descendents of these matings, and to determine whether an additional mutation induced by ENU will increase the level of breast cancer susceptibility.

A total of 68 sublines derived by this strategy are currently being observed. No increase in tumor susceptibility has been observed in any of the lines so far, although most of them have been established so recently that this result is not unexpected. Two phenotypes were observed that may have resulted from new mutations, but they were unrelated to breast cancer. No sequence differences have been identified yet that could be attributed to the ENU treatment.

The most interesting observation however, has to do with the MN-10 parental line. This is a transgenic line with a BALB/c background that was derived by introducing a c-neu oncogene under the control of the MMTV promoter into the genome. The degree of breast cancer susceptibility of these mice has been reported in the literature (Katsumata et al., Nature Medicine 1: 644-648, 1995). As the experiment progressed, it became apparent that this line no longer has the same degree of susceptibility as what we observed approximately 7 years previously. As it has been maintained as a small closed colony, genetic changes would appear to be an unlikely explanation. However, during that period of time, the conditions under which these mice were maintained were dramatically improved. We are considering the possibility that bacterial or viral infections, or other environmental conditions, may have had an impact on the breast cancer susceptibility of this transgenic line

Table 1. Numbers of MN-10 mice that had breast tumors or did not have breast tumors by 52 weeks of age in conventional animal facilities vs. SPF facilities.

	Conventional Facilities	SPF Facilities
Tumors	20	0
No Tumors	3	6

$X^2 = 16.8$ $P < 0.001$

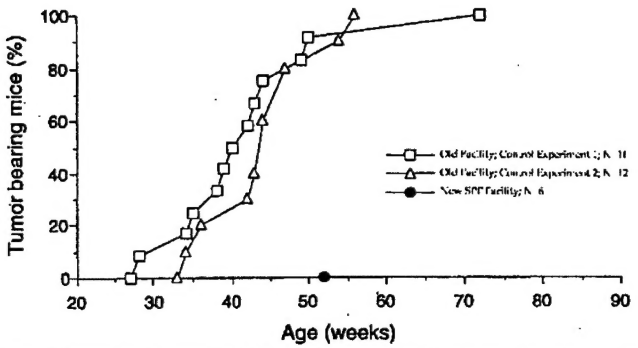

Figure 1. Frequency of tumors in MN-10 mice maintained in old facilities with minimal pathogen control and those in new specific pathogen-free (SPF) facilities.

Table 2. Possible risk factors that were present in the old facilities

- Viruses
  - -Mouse Hepatitis Virus (MHV)
  - -Pneumonia Virus of Mice (PVM)
  - -Minute Virus of Mice (MVM)
  - -GDVII (a strain of Theiler's Virus)
  - -Sendai Virus
  - -Mouse Adenovirus
- Mites
- Pinworms
- Changes in bedding
- Differences in drinking water
- Ammonia concentration
- Stress level

Conclusions

An experiment has been initiated to search for ENU-induced mutations in mice that will interact with the c-neu oncogene to increase the susceptibility of mice to breast tumors. No increases have been observed yet, although 68 sublines that were recently established are now being observed.

A transgenic line of mice that has the c-neu oncogene under the control of the MMTV promoter is one of the parental control lines. The susceptibility of this line (MN-10) to the development of mammary tumors appears to be less than what was observed approximately 7 years previously. During the intervening period, SPF colonies were established and this line was re-derived for entry into those facilities. We are considering the possibility that a significant improvement in animal care conditions had an effect on this phenotype.

Research Funded by the Breast Cancer Research Program of the United States Army Research and Materiel Command, Award Number DAMD17-00-1-0398.

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Table 1. Numbers of mice that had breast tumors and did not have tumors by 52 weeks of age in old facilities vs. SPF facilities.

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$X^2 = 16.8$ $P < 0.001$

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- **Mouse Adenovirus**

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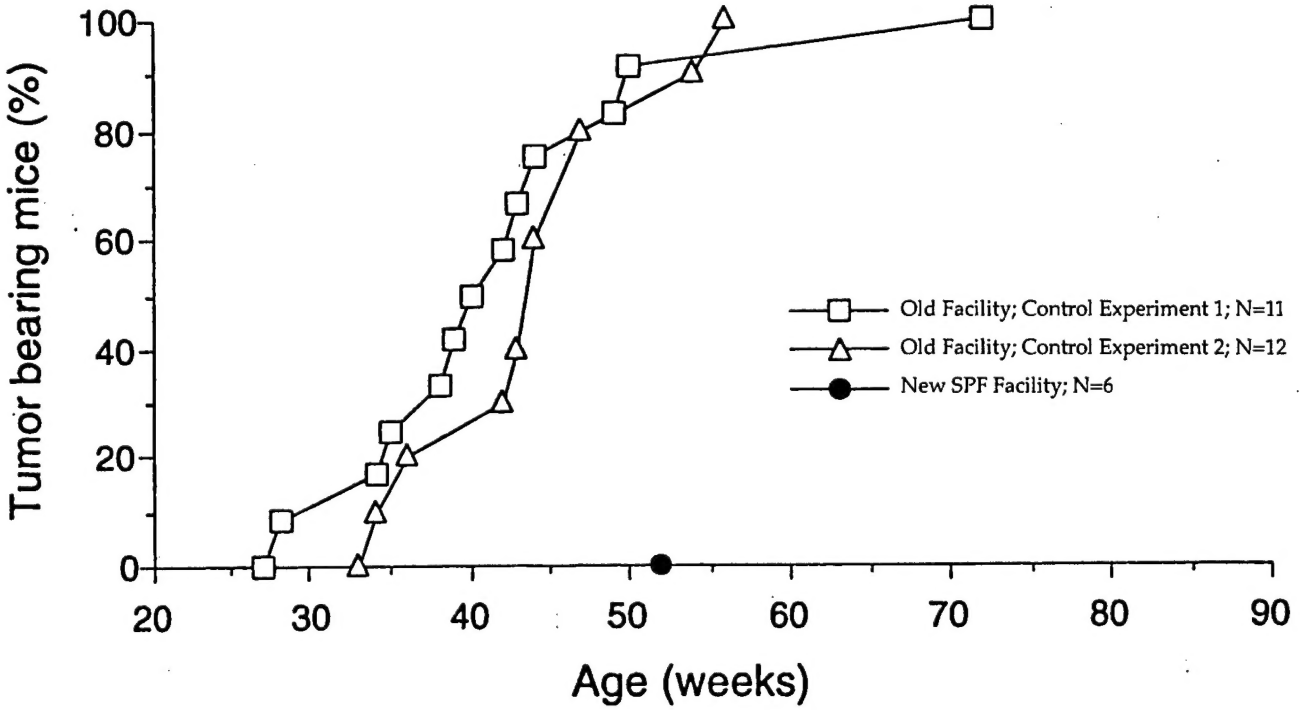
Pinworms

Changes in bedding

Differences in drinking water

Ammonia concentration

Stress level



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